JOURNAL OF VIROLOGY, June 1993, p. 3470–3480
0022-538X/93/063470-11$02.00/0

Vol. 67, No. 6

Herpes Simplex Virus Type 1 DNA Cleavage and Encapsidation Require the Product of the UL28 Gene: Isolation and Characterization of Two UL28 Deletion Mutants

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Received 5 January 1993/Accepted 3 March 1993

The herpes simplex virus type 1 UL28 gene contains a 785-amino-acid open reading frame that codes for an essential protein. Studies with temperature-sensitive mutants which map to the UL28 gene indicate that the UL28 gene product (ICP18.5) is required for packaging of viral DNA and for expression of viral glycoproteins on the surface of infected cells (C. Addison, F. J. Rixon, and V. G. Preston, J. Gen. Virol. 71:2377–2384, 1990; B. A. Pancake, D. P. Aschman, and P. A. Schaffer, J. Virol. 47:568–585, 1983). In this study, we describe the isolation of two UL28 deletion mutants that were constructed and propagated in Vero cells transformed with the UL28 gene. The mutants, gCB and gCΔ7B, contained deletions of 1,881 and 537 bp, respectively, in the UL28 gene. Although the mutants synthesize viral DNA, they fail to form plaques or produce infectious virus in cells that do not express the UL28 gene. Transmission electron microscopy and Southern blot analysis demonstrated that both mutants are defective in cleavage and encapsidation of viral DNA. Analysis by cell surface immunofluorescence showed that the UL28 gene is not required for expression of viral glycoproteins on the surface of infected cells. A rabbit polyclonal antiserum was made against an *Escherichia coli*-expressed Cro-UL28 fusion protein. This antibody reacted with an infected-cell protein having an apparent molecular mass of 87 kDa. The 87-kDa protein was first detected at 6 h postinfection and was expressed as late as 24 h postinfection. No detectable UL28 protein was synthesized in gCB- or gCΔ7B-infected Vero cells.

The herpes simplex virus type 1 (HSV-1) genome is 152,260 bp in size and contains at least 72 open reading frames (27). Genetic and biochemical evidence suggests that at least 30 of the proteins encoded by these open reading frames are components of the HSV-1 virion (6, 17, 26, 38, 48). The architecture of the HSV-1 virion consists of a trilaminar lipid envelope, an amorphous layer known as the tegument, an icosohedral capsid, and a DNA-containing core (11, 42, 47, 51). The HSV-1 capsid is assembled in the nuclei of virus-infected cells, and seven polypeptides have been identified as capsid components (7, 11, 13, 16, 17). The seven capsid proteins are the products of six HSV-1 genes. VP5, VP19C, VP22a, VP23, VP24, and VP26 are the products of genes UL19, UL38, UL26.5, UL18, UL26, and UL35, respectively (8, 10, 24, 28, 29, 37, 39–41, 43, 50). VP21 is probably the product of the UL26 gene, but this assignment has not been directly confirmed (8). Three different capsid types, designated A, B, and C or empty, intermediate, and full, respectively, have been identified in wild-type virus-infected cells (4, 13, 34–36, 44). Type A capsids are intranuclear capsids devoid of DNA and internal structure. Type B capsids are also intranuclear capsids that lack DNA but exhibit internal structural features. Type C capsids contain DNA and a densely staining core and are the capsids which are enveloped and released from infected cells.

In addition to the six HSV-1 genes required for capsid assembly, at least five HSV-1 genes that are required for cleavage and encapsidation of viral DNA have been identified. Studies with HSV-1 temperature-sensitive (*ts*) mutants have shown that the products of the UL6, UL25, UL28, UL32, and UL33 genes are required for formation of full capsids (1–3, 45, 46). At the nonpermissive temperature these mutants synthesize near-wild-type levels of viral DNA but are defective in nucleocapsid maturation, accumulating only type B capsids, and the viral DNA remains in a high-molecular-weight (concatemeric) form. The DNA synthesized at the nonpermissive temperature by these *ts* mutants can be cleaved and packaged after shifting to the permissive temperature but this is dependent on the synthesis of new proteins and capsids following the temperature shift. These results indicate that the cleavage of viral DNA and its encapsidation are tightly coupled events. In addition, these results are consistent with a model of nucleocapsid assembly in which empty capsids are filled with concatemeric DNA which is cleaved when a genome equivalent is packaged into the capsid.

The UL28 gene is a highly conserved gene within the herpesvirus family. Homologs have been found in pseudorabies virus, HSV-2, Epstein-Barr virus, human cytomegalovirus, varicella-zoster virus, equine herpesvirus, Marek's disease virus, bovine herpesvirus, and herpesvirus saimiri (reviewed in reference 32). A 95-kDa protein has been identified in HSV-1-infected cells by using rabbit antiserum made against a synthetic peptide that was synthesized from the predicted sequence of the UL28 protein (33). In pseudorabies virus, the UL28 homolog has been shown to be a 79-kDa protein that plays an essential role in the cleavage and encapsidation of viral DNA (30, 33). Two different *ts* mutants have been isolated, which map within the UL28 gene of HSV-1 (2, 31), and two very different phenotypes have been described for these two mutants. *ts*Z47 fails to express viral glycoproteins on the surface of infected cells,

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whereas *ts*1203 is defective in cleavage and encapsidation of viral DNA. To further investigate the role of the UL6, UL25, UL28, UL32, and UL33 genes in cleavage and encapsidation of viral DNA, we have set out to construct additional mutants with mutations in these genes. In this paper, we describe the isolation and characterization of two UL28 null mutants. The mutants were propagated on cell lines expressing the wild-type UL28 gene, and the phenotype of the UL28 mutants was characterized. In this report, we identify the UL28 gene product and demonstrate that it is required for cleavage and encapsidation of viral DNA but that it is not needed for cell surface expression of viral glycoproteins.

(These results were presented at the 17th International Herpesvirus Workshop, Edinburgh, Scotland, August 1992.)

MATERIALS AND METHODS

Cells, viruses, and monoclonal antibodies. African green monkey kidney cells (Vero; ATCC CCL 81) were propagated and maintained as previously described (21). Vero-A1 (A1), Vero-C1 (C1), and Vero-D6 (D6) cells were propagated and maintained in Dulbecco modified Eagle medium plus 10% fetal bovine serum and 0.5 mg of G418 (Geneticin; GIBCO-BRL) per ml. HSV-1 KOS was used as the wild-type strain and HSV-1 Δ2 (22) was used as the parental virus for the isolation of the UL27 and UL28 host range mutants. HSV-1 gB deletion mutant KΔT and the gB-complementing cell line D6 were provided by Stan Person, University of Pittsburgh Medical School, Pittsburgh, Pa. (5). Pools of gB-, gC-, and gD-specific monoclonal antibodies were obtained from Joseph Glorioso, University of Pittsburgh Medical School. The gB pool contained antibodies B4, B7, and B8 (25). The gC antibodies (pool 2) have been described previously (20). The gD pool contained antibodies D6, D8, and D9 (18).

Plasmids. pRSV-neo contains the bacterial gene for neomycin resistance under control of the Rous sarcoma virus promoter (14). Plasmid pSG18-B50 (the plasmid used to isolate cell line A1; see below and Fig. 1) containing sequences of the HSV-1 genome between bases 52588 and 60362 was described previously (19). Plasmid pSG18-BΔ50 (the plasmid used to isolate cell line C1, see below and Fig. 1) is identical to pSG18-B50 with the exception that a 969-bp *Bst*EII fragment (nucleotides 53164 to 54133) was deleted by partially digesting pSG18-B50 with *Bst*EII and then religating it with T4 DNA ligase.

Plasmids pSG18-gCΔ7B and pSG18-gCB were constructed from pSG18-B50 in the following manner. pSG18-B50 was digested with *Xho*I, and the ends were blunted with T4 DNA polymerase. Following ligation of *Bgl*II linkers, the sample was digested with *Bgl*II and *Xho*I; the vector fragment generated by this digest was gel purified and ligated with a 268-bp *Bgl*II-*Xho*I fragment of the HSV-1 gC gene (−144 to +124 region of the gC promoter) isolated from a *Bgl*II-*Xho*I digest of plasmid pGCΔ7 (22) to yield pSG18-gCΔ7B. pSG18-gCB was constructed by digesting pSG18-gCΔ7B with *Bgl*II and subjecting the product to a partial digest with *Bst*EII; the appropriate vector fragment generated by this digest was isolated and ligated with a 693-bp *Bgl*II-*Bst*EII fragment of the HSV-1 gC gene (the −569 to +124 region of the gC promoter) isolated from a *Bst*EII-*Bgl*II digest of plasmid pGC (22).

The *Escherichia coli* expression plasmid pECH85 contains a gene fusion consisting of 27 codons of the λcro protein, 30 codons from the 5′ noncoding region of the UL28 gene, and all 786 codons of the UL28 gene. Transcription is under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible Lac promoter, and translation terminates at the UL28 TAG stop codon. pECH85 was constructed in the following manner. A 1.47-kb *Sma*I fragment containing the 5′ end of the HSV-1 UL28 gene was isolated from pKBG (9) and ligated into the *Hinc*II site of pBlueScript SK(−) (Stratagene, La Jolla, Calif.) to generate pECH76. The 3′ end of the UL28 gene was added as a 2.77-kb *Bst*EII-*Fsp*I fragment to the unique *Bst*EII and *Eco*RV sites of pECH76 to generate pECH82. A 3.1-kb *Bam*HI-*Cla*I fragment isolated from pECH82 was then cloned into the unique *Bam*HI and *Cla*I sites of pHK412 (32) to generate pECH85.

Transformation of Vero cells. At 1 day prior to transfection, approximately 2×10^6 Vero cells were seeded in 100-mm-diameter culture dishes. Cells were cotransformed with calcium phosphate-precipitated DNA (6 μg of pSG18-B50 or pSG18-BΔ50, 0.5 μg of pRSV-neo, and 20 μg of salmon testis DNA) by the method of Graham and van der Eb (15). After a 4-h incubation at 37°C, the medium was removed and the cells were glycerol shocked (15% glycerol). The transfected cells were left in normal growth medium for 48 h, trypsinized, and split 1:10 into additional 100-mm-diameter culture dishes containing medium with 1 mg of G418 per ml. After 14 to 21 days, individual G418-resistant colonies were isolated with cloning cylinders. Ten clonal isolates from cells transfected with pSG18-B50 (cell lines A1 to A10) or pSG18-BΔ50 (cell lines C1 to C10) were amplified, and aliquots were frozen.

Isolation of recombinant viruses. Marker transfer of mammalian cells with HSV-1 genomic DNA and cloned DNA fragments was done as described previously (22). Infectious Δ2 viral DNA was mixed with a fivefold molar excess of a cloned DNA fragment containing a disrupted UL28 gene and used to transfect A1 or C1 cells. The resulting virus stocks were screened for the ability to hybridize with a restriction fragment specific for the region deleted in the Δ2 virus by an in situ hybridization procedure (22). The Δ2 virus contains a deletion that removes sequences of the gC gene between bases −569 and +124 relative to the 5′ terminus of the gC mRNA. The two plasmids used in the cotransfections, pSG18-gCB and pSG18-gCΔ7B, contain a portion of the sequences that are deleted from the Δ2 virus. Hybridizing plaques were purified three times on C1 cells before stocks were made.

DNA isolation. Total-infected-cell DNA and DNase I-treated total-infected-cell DNA were harvested by the method of Stow et al. (49). Cells were infected at a multiplicity of infection (MOI) of 5. At approximately 3 days postinfection, cells were removed into a 50-ml conical centrifuge tube and spun at 2,000 rpm (Sorvall SS34 rotor) for 15 min. The cell pellet was frozen and thawed once, resuspended in 1 ml of lysis buffer (0.6% sodium dodecyl sulfate [SDS], 10 mM EDTA, 10 mM Tris-HCl [pH 7.5], 500 μg of proteinase K per ml), and held at 37°C for 4 h. NaCl was added to a final concentration of 200 mM, and the sample was extracted once with phenol and once with phenol-chloroform, and the DNA was ethanol precipitated. The final DNA pellet was resuspended in TE buffer (10 mM Tris-HCl [pH 7], 1 mM EDTA) containing 10 μg of RNase A per ml. For preparation of DNaseI-treated DNA, cells were infected and harvested as described above and the cell pellet was resuspended in 1 ml of rSb buffer (10 mM Tris-HCl [pH 7.4], 10 mM KCl, 1.5 mM $MgCl_2$) and sonicated, 50 μg of RQ1 RNase-free DNase (Promega) was added, and the sample was incubated at 37°C for 2 h. SDS, EDTA, and proteinase K were then added as described above, and the sample was

subjected to phenol-chloroform extraction and precipitation as described above.

HSV-1 DNA encapsidation. Cells were infected with virus at an MOI of 5 and harvested 3 days postinfection. Total-infected-cell DNA was prepared from half of the sample, and DNase I-treated total-infected-cell DNA was prepared from the other half (see above). All of the DNA samples were then digested with *Bam*HI, and Southern blots were prepared as previously described (22). The blots were probed with the ^{32}P-labeled *Bam*HI K fragment of HSV-1 DNA.

Analysis of viral DNA replication. Viral DNA synthesis was determined by the method of Gadler (12) as modified by Zhu and Weller (52). Cells in 75-cm^2 flasks were infected with virus at an MOI of 5 for 24 h at 37°C. The flasks were washed once with phosphate-buffered saline (PBS), and the cell monolayer scraped into 1 ml of PBS. The cells were pelleted and resuspended in 100 μl of PBS and freeze-thawed once. A series of fivefold dilutions in PBS were spotted onto GeneScreen Plus (NEN/Dupont) by using a dot blot apparatus (Schleicher & Schuell). The membrane was treated twice with 0.4 M NaOH and twice with 1 M Tris (pH 8.0). The blot was probed with a ^{32}P-labeled HSV-specific probe (*Sal*I fragment, nucleotides 54826 to 58701, isolated from a *Sal*I digest of pSG18-B50 [see Fig. 1]) as previously described (22).

Transmission electron microscopy. Monolayers of either Vero or C1 cells were grown to 90% confluency and infected with either KOS, gCB, or gCΔ7B at an MOI of 5. At 12 h postinfection, the cells were fixed for 30 min with 1% glutaraldehyde–0.1 M sodium cacodylate buffer (Nacac) and rinsed three times for 10 min with 0.1 M Nacac–7% sucrose. Samples were held in this buffer overnight at 4°C and then postfixed with 1% osmium tetroxide for 1 h at room temperature. The samples were rinsed again in 0.1 M Nacac–7% sucrose and embedded in epon resin. Sections 1 μm thick were prepared, stained with toluidine blue, and observed by light microscopy for areas appropriate for thin sectioning. Samples were then thin sectioned and prepared by contrast staining with lead citrate and uranyl acetate. Thin sections were visualized on a JEOL 1200ES transmission electron microscope and subsequently photographed. A minimum of 10 fields were visualized for each sample.

Immunofluorescence. Vero and C1 cells were grown to confluency on 35-mm dishes. The cells were infected at an MOI of 2, and at 8 h postinfection they were rinsed with PBS three times. They were briefly fixed with freshly prepared 3% paraformaldehyde for 10 min at room temperature and rinsed three times with PBS and once with PBS-BLOTTO (BLOTTO is 2% nonfat dry milk plus 1% bovine serum albumin. Primary antibody (a pool of either gC or gD monoclonal antibodies) was applied at a 1:1,000 dilution in PBS-BLOTTO for 45 min at 37°C. Monolayers were then gently rinsed twice with 1× BLOTTO, twice with 0.5× BLOTTO, and then once with PBS alone. Fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin G (Sigma) was applied at a concentration of 1:128 in 1× PBS-BLOTTO and the cells were incubated for 1 h at room temperature (in the dark) and then rinsed three times with PBS. Just prior to observation, the samples were rinsed with a freshly prepared antiquench reagent, a 1-mg/ml *p*-phenylenediamine solution made in PBS–20% glycerol (23). Coverslips were applied, and the fluorescence was observed and recorded by fluorescence photomicroscopy.

Western immunoblots and UL28-specific antisera. Proteins were electroblotted onto 0.2-μm polyvinylidene difluoride membranes (Transblot; Bio-Rad, Richmond, Calif.) as previously described (32). The Western-Light chemiluminescent detection system (Tropix, Inc., Bedford, Mass.) was used to identify proteins reactive with the H85 antisera. The filters were blocked overnight at 4°C in PBS containing 5% powdered milk. Then H85 antiserum (diluted 1:200) was added to the blocking solution and incubated for 2 h at room temperature. The filters were subsequently incubated with alkaline phosphatase-conjugated immunoglobulin G in PBS with 5% powdered milk, washed, and incubated with the dioxetane substrate CSPD, and chemiluminescence was detected by exposure of the membrane to Kodak X-Omat XAR5 film (Kodak, Rochester, N.Y.). The H85 antiserum was raised against a Cro-UL28 fusion protein expressed from *E. coli* by using plasmid pECH85 (see above). The Cro-UL28 protein accumulated as insoluble aggregates in *E. coli* NFPU2 when induced with 1 mM IPTG. The aggregates were purified as previously described (32), emulsified in complete Freund's adjuvant, and injected intramuscularly into New Zealand White rabbits. Subsequent boosts were injected intramuscularly in Freund's incomplete adjuvant.

Immunoprecipitation. Immunoprecipitation was done as described previously (20), with a mixture of the gB and gD monoclonal antibodies. Antigen-antibody complexes were collected with protein A-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, N.J.).

RESULTS

Isolation of two UL28 deletion mutants. According to the DNA sequence analyses of McGeoch et al. (27), the 785-amino-acid open reading frame for the UL28 gene extends from nucleotide 58159 (ATG) to nucleotide 55802 (TAG) in the HSV-1 genome (Fig. 1). The 5.6-kb UL28 mRNA and the 3.3-kb UL27 mRNA are expressed as 3′-coterminal transcripts, and the promoter for the UL27 gene is contained within the C-terminal coding region of the UL28 gene (the transcription start site of UL27 gene is at nucleotide 56080). Two plasmids containing deletions in UL28 gene coding sequences were constructed starting from plasmid pSG18-B50 (Fig. 1). pSG18-gCB contains an 1,881-bp *Bst*EII-*Xho*I deletion (nucleotides 57923 to 56042) that removes the coding region for amino acids 78 to 706 of the UL28 gene, and pSG18-gCΔ7B contains a 537-bp *Xho*I-*Xho*I deletion (nucleotides 56579 to 56042) that removes the coding region for amino acids 528 to 706 of the UL28 gene. The deletions introduced into pSG18-gCB and pSG18-gCΔ7B remove essential promoter sequences from the UL27 gene. To ensure that these two deletions did not alter expression of the UL27 gene, the deleted sequences were replaced with sequences of the HSV-1 gC promoter (Fig. 1). The gC promoter fragment inserted into pSG18-gCB consisted of a 693-bp *Bst*EII-*Bgl*II fragment that includes sequences located 569 bp upstream and 124 bp downstream of the gC gene transcription start site, whereas the gC promoter fragment inserted into pSG18-gCΔ7B consisted of a 268-bp *Xho*I-*Bgl*II fragment that includes sequences located 144 bp upstream and 124 bp downstream of the gC gene transcription start site. Both of the gC promoter fragments have been previously shown to contain complete elements of the gC promoter (22).

The UL28 gene of HSV-1 is essential for viral replication (2). To propagate viral mutants with deletions in the UL28 gene, we constructed cell lines which express the wild-type UL28 gene. To select for cell lines that carry the UL28 gene, we relied on the fact that the UL28 gene overlaps at its 3′ end with the essential UL27 (gB) gene (Fig. 1). Vero cells were cotransformed with plasmids pRSV-neo, which contains the

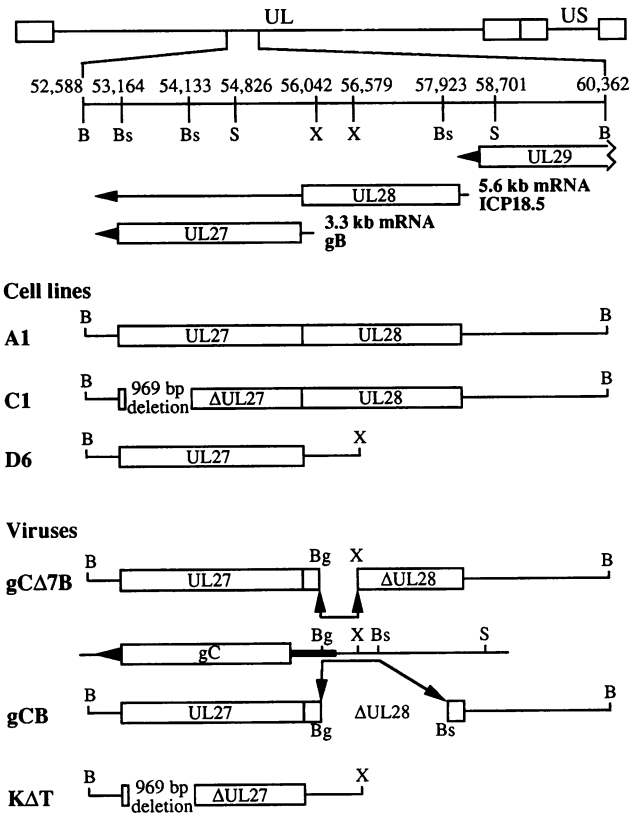


FIG. 1. Regions of the HSV-1 genome that were used to isolate transformed cells and recombinant viruses. The HSV-1 genome is shown at the top of the figure, with UL and US referring to the long and short unique region sequences, respectively. The *Bam*HI region located between nucleotides 52588 and 60362 of the HSV-1 genome is expanded to demonstrate the genomic location of the UL27 (gB) and UL28 (ICP18.5) genes (19, 27). The location of the UL27 and UL28 mRNA coding sequences are shown with the open boxed regions representing the open reading frames for these two genes. For the cell lines, regions of the HSV-1 contained in recombinant plasmids pSG18B50, pSG18BΔ50, and pKBXX are shown. These plasmids were used to isolate transformed Vero cell lines A1 (pSG18B50) C1 (pSG18BΔ50), or D6 (isolated by Cai et al. [5] by using pKBXX). For the viruses, plasmids pSG18-gCΔ7B and pSG18-gCB were constructed and used to isolate the two UL28 deletion viruses gCΔ7B and gCB as described in Materials and Methods. pSG18-gCB contains an 1,881-bp deletion that removes nearly all of the coding region of the UL28 gene while pSG18-gCΔ7B contains a 537-bp deletion in the 3′ end of the UL28 gene. The deleted sequences were replaced with the indicated regions of the HSV-1 gC gene. In both recombinant viruses, expression of the UL27 gene is from the gC promoter sequences that were inserted. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; H, *Hin*dIII; S, *Sal*I; X, *Xho*I.

G418 resistance gene, and pSG18-B50, which contains both the UL27 (gB) and UL28 genes of HSV-1 (Fig. 1). After 2 weeks, 10 G418-resistant colonies were isolated, amplified, and screened for their ability to complement replication of the gB deletion mutant KΔT. One of these cell lines, designated A1, yielded the highest level of complementation and was chosen for further study.

The mutations in pSG18-gCB and pSG18-gCΔ7B were transferred into the viral genome by marker transfer in which A1 cells were cotransfected with each of the plasmids and HSV Δ2 viral DNA. The progeny from the transfection were

TABLE 1. Plaque-forming efficiencies of HSV-1 strains on Vero, A1, C1, and D6 cells[a]

Virus	Cell line	Titer (PFU/ml)
KOS	Vero	2.4×10^9
	A1	3.0×10^9
	C1	1.9×10^9
	D6	1.6×10^9
Δ2	Vero	1.3×10^9
	A1	1.5×10^9
	C1	0.9×10^9
	D6	1.0×10^9
KΔT	Vero	$<5 \times 10^2$
	A1	8.8×10^5
	C1	$<5 \times 10^2$
	D6	6.2×10^5
gCB	Vero	$<5 \times 10^2$
	A1	4×10^7
	C1	6.5×10^7
	D6	$<5 \times 10^2$
gCΔ7B	Vero	$<5 \times 10^2$
	A1	1×10^7
	C1	1×10^7
	D6	$<5 \times 10^2$

[a] Virus stocks were prepared in Vero cells (KOS and Δ2), D6 cells (KΔT), and C1 cells (gCB and gCΔ7B).

then plated on A1 cells and screened by a previously described, in situ viral plaque hybridization procedure (22) for the ability to hybridize with a restriction fragment specific for the region deleted in the Δ2 virus. The Δ2 virus contained a deletion that removed bases −569 to +124 of the gC gene (22). Because both pSG18-gCB and pSG18-gCΔ7B contain all or a portion of the −569 to +124 gC promoter sequences, homologous recombination between the plasmid and viral sequences resulted in the introduction of the gC sequences at the UL28 locus. The inserted sequences could then serve as a marker to screen for recombinant products. Positively hybridizing plaques were selected from the two transfections, plaque purified three times, and propagated on A1 cells. The recombinant viruses isolated from transfections with pSG18-gCB and pSG18-gCΔ7B were designated gCB and gCΔ7B, respectively.

To confirm that the gCB and gCΔ7B viruses harbored the UL28 mutations present in pSG18-gCB and pSG18-gCΔ7B, we tested the growth properties of these two mutants by measuring their plaque-forming efficiencies on A1, Vero, and D6 cells (Table 1). Both mutants were incapable of forming plaques in Vero or D6 cells, but both formed plaques equally well in A1 cells. KOS and Δ2 formed plaques on all three cell lines, whereas the gB deletion virus, KΔT, formed plaques in D6 and A1 cells but not in Vero cells. Because A1 cells are capable of complementing the gB deletion mutant, KΔT, we could not rule out that the gCB and gCΔ7B viruses harbored mutations which affected both the UL27 and UL28 genes. To address this question, we isolated a second UL28-expressing cell line, designated C1 (Fig. 1). The C1 cell line was isolated by cotransfecting Vero cells with plasmids pSG18-BΔ50 and pRSV-neo, and G418 resistant cell lines were isolated and tested for the ability to complement the growth of mutants gCB and gCΔ7B. Plasmid pSG18-BΔ50 is identical to pSG18-B50, except that a 969-bp *Bst*EII frag-

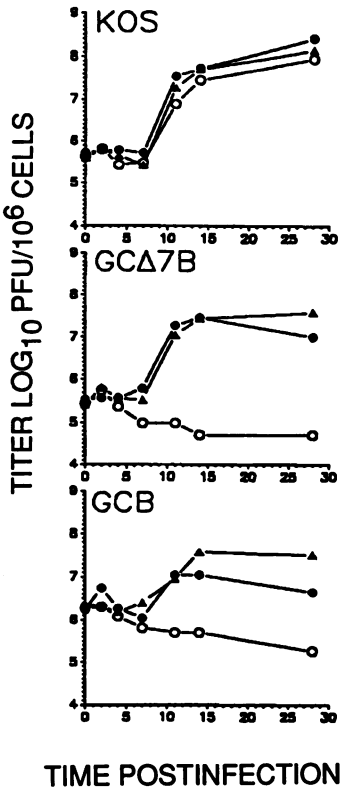


FIG. 2. Single-step growth of KOS, gCB, and gCΔ7B. Replicate cultures of Vero (○), A1 (▲), and C1 (●) cells were infected with KOS, gCB, or gCΔ7B at an MOI of 5. The cultures were harvested at the indicated times postinfection and freeze-thawed three times, and the yield of virus at each time point was determined by plaque titer determination on A1 cells.

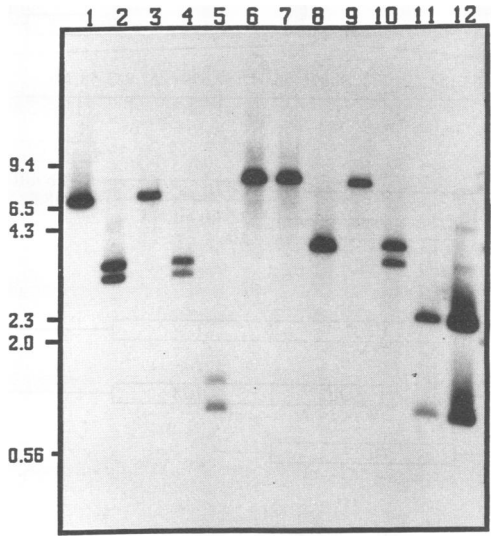


FIG. 3. Southern blots of DNA from UL28 deletion mutants. Autoradiographic exposure of a Southern blot of plasmid DNA or viral DNA separated on 0.8% agarose gels. Total-infected-cell DNA isolated from Vero cells infected with KOS (lanes 6 to 8), gCB (lanes 3 to 5), or gCΔ7B (lanes 9 to 11) and plasmid DNA from pSG18-gCB (lanes 1 and 2) and pSG18-gCΔ7B (lane 12) were digested with either *Bam*HI (lanes 1, 3, 6, and 9), *Bam*HI-*Bgl*II (lanes 2, 4, 7, and 10), or *Bam*HI-*Bgl*II-*Sal*I (lanes 5, 8, 11, and 12). The blot was hybridized with a UL28-specific probe (*Sal*I fragment, nucleotides 54826 to 58701, gel purified from a *Sal*I digest of pSG18B50 [Fig. 1]). Markers, *Hin*dIII digest of lambda DNA.

ment (nucleotides 53164 to 54133) has been deleted (Fig. 1). The *Bst*EII deletion removes sequences that code for the membrane-spanning domain of the gB protein (the same deletion as in the KΔT virus) and results in the expression of a secreted form of the gB protein. When we tested the plaque-forming efficiencies of KOS, Δ2, gCB, gCΔ7B, and KΔT on C1 cells, we found that KΔT was the only virus which failed to form plaques in these cells (Table 1). The nearly 100-fold difference in titers of KOS and Δ2 compared with the mutant viruses is probably the result of low levels of complementation by the complementing cell lines. Taken together, the data from the plaque assays with A1 (complements both UL27 and UL28 mutants), C1 (complements UL28 mutants), and D6 (complements UL27 mutants) cells demonstrate that both gCB and gCΔ7B fail to plaque on Vero cells because of a UL28 mutation. Intracellular replication of the two UL28 deletion mutants was examined by establishing single-step growth curves for KOS, gCB, and gCΔ7B in Vero, A1, and C1 cells. Samples from each infected culture were harvested at various times postinfection and assayed for infectious virus by determination of virus titer on A1 cells. All three viruses replicated in A1 and C1 cells, and the time course of replication was the same for each virus (Fig. 2). Only KOS replicated on Vero cells, indicating that the UL28 deletion mutants fail to replicate (produce infectious virus particles) in cells that do not express the UL28 protein. The parent virus Δ2 showed similar growth to that of KOS on all three cell lines (data not shown).

To demonstrate that the two UL28 mutants contain the deletion/insertion mutations at the appropriate position in the viral genome, we isolated total-cell DNA from Vero cells infected with KOS, gCB, or gCΔ7B. The DNA samples were digested with either *Bam*HI, *Bam*HI-*Eco*RI, or *Bam*HI-*Eco*RI-*Sal*I, and subjected to gel electrophoresis and Southern blot hybridization (Fig. 3). Plasmids pSG18-gCB and pSG18-gCΔ7B were also digested with some of the same enzymes and subjected to electrophoresis in parallel with the viral DNA. The filter was probed with a 3,839-bp *Sal*I fragment (nucleotides 54826 to 58701 [Fig. 1]) that contains the entire UL28 gene and a portion of the UL27 gene. The *Bam*HI digests demonstrate that both gCB and gCΔ7B contain *Bam*HI fragments (Fig. 3, lanes 3 and 9) that are smaller than the KOS fragment (lane 6) and that the gCB *Bam*HI fragment is identical in size to the *Bam*HI insert of pSG18-gCB (compare lanes 1 and 3). The *Bam*HI-*Bgl*II digests demonstrate that the two mutants contain a *Bgl*II site near the center of the *Bam*HI fragment, which is not present in the same *Bam*HI fragment from KOS DNA (lanes 4, 7 and 10) and that identical-sized *Bam*HI-*Bgl*II fragments are detected in pSG18-gCB (lane 2) and gCB (lane 4). Finally, the *Bam*HI-*Bgl*II-*Sal*I digests clearly shows that the gCΔ7B (lane 11) virus contains the same hybridizing fragments as pSG18-gCΔ7B (lane 12). We concluded from these Southern blot data that the desired UL28 deletion mutants had been generated.

Replication, cleavage, and packaging of HSV-1 DNA. Previous studies with a *ts* mutant (*ts*1203) whose mutation maps to the UL28 gene showed that the UL28 gene was needed for cleavage and encapsidation of viral DNA (2). At the nonpermissive temperature, *ts*1203 was found to synthesize concatemeric viral DNA, which was not processed into unit

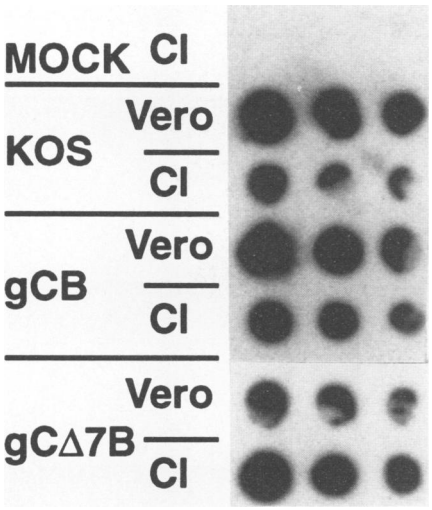


FIG. 4. Analysis of viral DNA synthesis. Vero cells or C1 cells were infected with the indicated virus at 37°C for 24 h. A series of fivefold dilutions were spotted on a nylon membrane, and the cells were lysed on the membrane. The ^{32}P-labeled *Sal*I fragment described in the legend to Fig. 2 was used as a hybridization probe, and the blots were visualized by autoradiography.

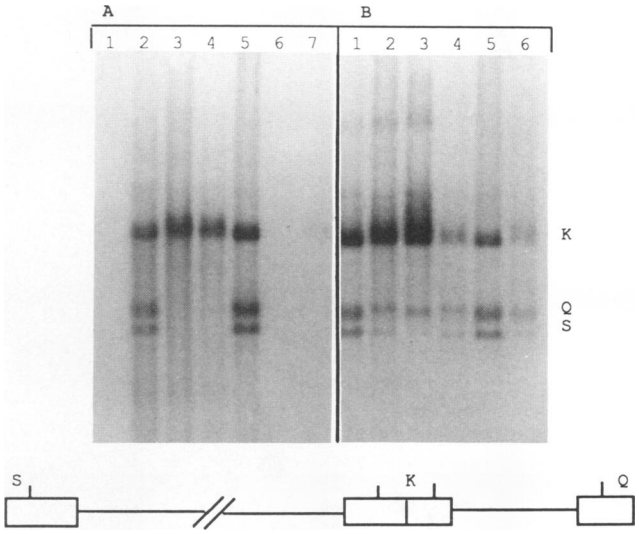
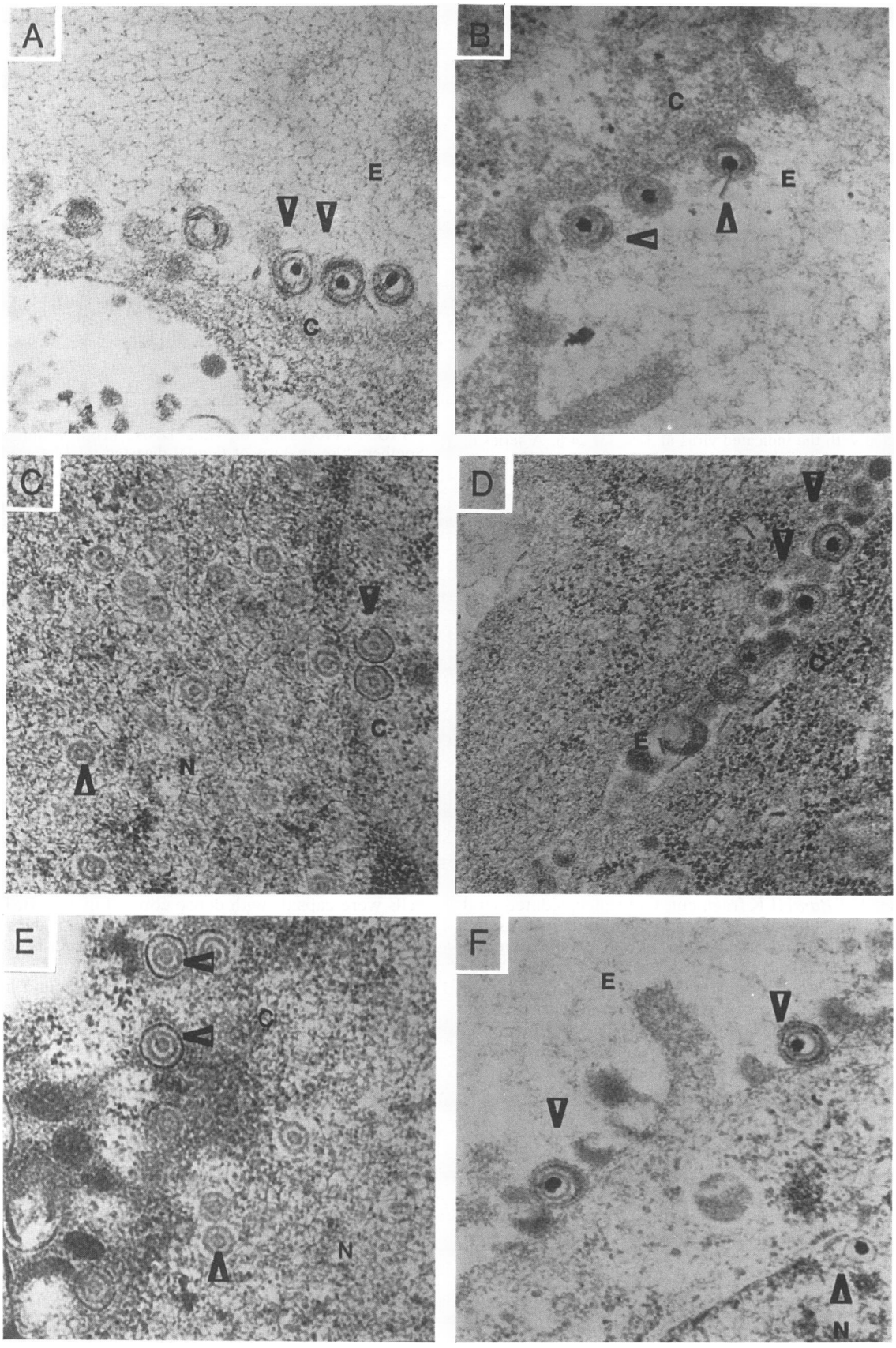


FIG. 5. Processing of virus DNA. (Top) Autoradiograph of Southern blot analysis of *Bam*HI-digested total-infected-cell DNA and encapsidated (DNase-resistant) DNA hybridized to the ^{32}P-labeled *Bam*HI K fragment of HSV-1 DNA. (A) DNA isolated from Vero cells mock infected (lane 1) or infected with KOS (lanes 2 and 5), gCB (lanes 3 and 6), or gCΔ7B (lanes 4 and 7). Lanes: 1 to 4, total-virus-infected-cell DNA; 5 to 7; encapsidated DNA. (B) DNA isolated from C1 cells infected with KOS (lanes 1 and 4), gCB (lanes 2 and 5), or gCΔ7B (lanes 3 and 6). Lanes: 1 to 3, total-virus-infected-cell DNA, 4 to 6, encapsidated DNA. (Bottom) HSV-1 genome, showing the locations of *Bam*HI-K, -Q, and -S fragments.

length molecules. The ability of gCB and gCΔ7B to induce viral DNA synthesis was assessed by dot blot hybridization of total DNA from infected cells to a virus-specific probe (see Materials and Methods). The results shown in Fig. 4 clearly demonstrate that gCB and gCΔ7B synthesize viral DNA in both C1 and Vero cells. These results indicate that the UL28 gene is not required for HSV DNA synthesis. To determine whether the newly synthesized viral DNA was cleaved and encapsidated, viral DNA processing was investigated by using Southern blot analysis. During viral DNA replication, concatemeric DNA is cleaved into unit-length molecules and packaged into virions. The encapsidated viral DNA contains free chromosomal termini, whereas the nonpackaged DNA does not. The presence of chromosomal ends can easily be monitored by Southern blot analysis of total-infected-cell DNA digested with *Bam*HI and probed with the HSV-1 *Bam*HI K fragment. Only encapsidated viral DNA with free chromosomal ends will give rise to the terminal *Bam*HI Q and S fragments, whereas concatemeric DNA gives rise only to the joint-spanning *Bam*HI K fragment (Fig. 5). Terminal fragments Q and S were absent from viral DNA isolated from Vero cells infected with the two UL28 mutants (Fig. 5A, lanes 3 and 4). When the infections were done with C1 cells, the terminal Q and S fragments were readily detected (Fig. 5B, lanes 2 and 3), indicating that the UL28 gene was required for efficient cleavage of concatemeric DNA to unit-length molecules. To examine the packaging of viral DNA, we treated infected-cell lysates with DNase I and then analyzed them by Southern blot analysis following *Bam*HI digestion (see Materials and Methods). Protection of encapsidated DNA from DNase I digestion is evidenced by the presence of the *Bam*HI K, Q, and S fragments. Vero cells infected with gCB or gCΔ7B do not contain DNase-resistant DNA (Fig. 5A, lanes 6 and 7), whereas the identical infections in C1 cells contain DNase-resistant DNA (Fig. 5B, lanes 5 and 6). These results clearly demonstrate that viral DNA synthesized in Vero cells infected with the two UL28 deletion mutants is endless and is not packaged into capsids.

Transmission electron microscopy of virus infected cells. Thin sections of virus-infected Vero and C1 cells were examined by transmission electron microscopy to determine the presence of full, intermediate, and empty capsids. The nuclei of Vero cells infected with either gCB or gCΔ7B contained capsids which had electron-translucent cores (intermediate capsids) (Fig. 6C and E). No dense-cored (full capsids) or empty capsids were observed in mutant-infected Vero cells. By contrast, the predominant capsid form found in mutant-infected C1 cells or KOS-infected Vero and C1 cells were capsids with dense cores (Fig. 6A, B, D, and F). These results corroborate the Southern blot data described above, showing that the UL28 gene product is required for assembly of full capsids.

Large numbers of virus particles were found in both the nucleus and cytoplasm of Vero and C1 separately cells infected with all three viruses. The intermediate capsids present in the cytoplasm of Vero cells infected with the two mutants appeared to be enveloped, although the envelope was distinctly different from the envelopes found in wild-type-virus-infected Vero or C1 cells or the envelopes found in C1 cells infected with either of the two mutants. The particles found in the cytoplasm of Vero cells infected with gCB and gCΔ7B appeared to contain a very defined envelope around the capsid, whereas wild-type virus and the mutants in C1 cells contained capsids that had a surrounding membrane but also contained an electron-opaque amorphous region beneath the envelope. The significance of the qualitative difference in the envelope found with virions made by the two mutants in Vero cells is unclear. However, the two UL28 null mutants differ in this regard from the UL28 *ts* mutants, since cytoplasmic, enveloped particles were not found at the nonpermissive temperature with the *ts* mutants.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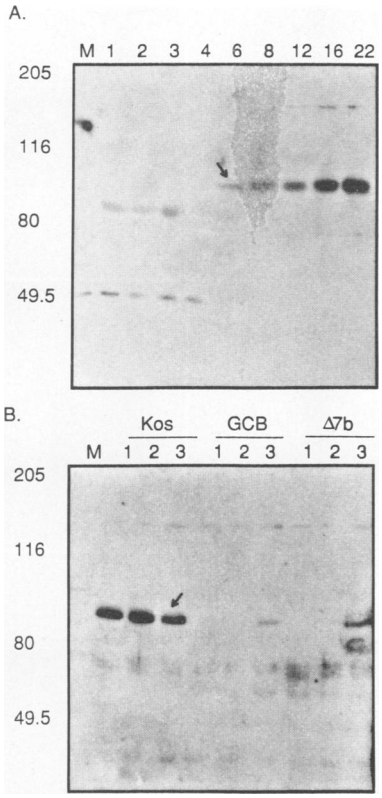

FIG. 7. Detection of the UL28 gene product during viral infection by immunoblotting with a chemiluminescence detection system (see Materials and Methods). (A) Time course of expression from KOS-infected Vero cells. Cells were harvested at the indicated times postinfection (time in hours given above each lane), and cell extracts were prepared as described in Materials and Methods and subjected to electrophoresis and immunoblotting. Lane M, mock-infected Vero cells. (B) Expression of the UL28 protein from mutant-infected cells. Vero cells (lanes 1 and 2) or C1 cells (lanes 3) infected with the indicated virus were harvested at 12 h (lanes 1) or 22 h (lanes 2 and 3) postinfection and treated as described above. Filters were probed with polyclonal antisera raised against an *E. coli*-expressed Cro-UL28 fusion protein. Lane M, mock-infected C1 cells. The position of the UL28 protein is indicated with an arrow in both panels.

Detection of UL28 protein in KOS-, gCB-, and gCΔ7B-infected Vero and C1 cells. To identify the protein product of the HSV-1 UL28 gene, we raised a polyclonal antiserum (H85) against an *E. coli*-expressed Cro-UL28 fusion protein (see Materials and Methods). The H85 antiserum was used for immunoblot analysis of KOS-infected Vero cell extracts. In mock-infected cells and in infected cells harvested at 1, 2, 3, and 4 h postinfection, there was no specific reactivity by the antiserum (Fig. 7A). An immunoreactive band of 87 kDa was detected at 6 h postinfection, and the intensity of this band increased at later times of infection. The open reading frame of the UL28 gene predicts a protein of approximately 85 kDa, which is in good agreement with the size of the protein detected with the H85 antiserum. Two additional proteins of approximately 50 and 80 kDa were detected with the H85 antiserum at early times postinfection. The 50-kDa protein was a cell-expressed protein, since it was detected in mock-infected cells. The significance of the 80-kDa protein is not known.

To characterize expression of the UL28 protein from the two mutant viruses, we isolated extracts of infected Vero and C1 cells at 12 and 22 h postinfection. Neither gCB or gCΔ7B expressed an immunoreactive protein in Vero cells (Fig. 7B). This is expected for the gCB mutant, since the deletion removes nearly all of the UL28 coding sequences. gCΔ7B is predicted to encode a UL28 peptide of at least 60 kDa; however, a band of this size was not observed (Fig. 7B), indicating that either this protein is not recognized by the H85 antiserum or the truncated protein may not be stable. In gCB- and gCΔ7B-infected C1 cells, a protein identical in size to the UL28 protein expressed from KOS-infected Vero or C1 cells was detected (Fig. 7B). This protein was not present in mock-infected C1 cells (Fig. 7B, lane M). These results demonstrate that although the two mutants do not make a UL28 protein in infected Vero cells, they are capable of inducing expression of the UL28 gene resident in C1 cells. Therefore, this experiment confirms that the ability of C1 cells to support growth of the two UL28 null mutants is due to its ability to provide the missing UL28 protein.

Is the UL28 gene required for expression of viral glycoproteins on the surface of infected cells? Studies with a *ts* mutant, *ts*Z47, indicated that the UL28 gene may be required for efficient transport of viral glycoproteins in infected cells (31). Complementation analysis with HSV-1 and HSV-2 *ts* mutants indicated that the *ts* lesion was within the HSV-1 UL28 gene, but marker rescue experiments showed that two different regions of the HSV-1 genome could rescue the *ts* phenotype. To address the role of the UL28 protein in expression of viral glycoproteins, we infected Vero cells with either KOS or gCB and added [^{35}S]methionine to the cells at 5 h postinfection. At 24 h postinfection, the cells were harvested and lysed and expression of gB and gD were analyzed by immunoprecipitation with gB- and gD-specific monoclonal antibodies (Fig. 8). No difference in the relative amount of gB or gD expressed from gCB as compared with KOS was found. In addition, the sizes of the two viral glycoproteins were identical to that found in KOS-infected cells, indicating that mature glycoproteins were being expressed in gCB-infected Vero cells. To determine whether the glycoproteins were being expressed on the surface of infected cells, we performed cell surface immunofluorescence (Fig. 9). Vero cells infected with KOS (Fig. 9A and B), gCB (Fig. 9C and D), or gCΔ7B (Fig. 9E and F) were fixed at 8 h postinfection and treated with gD-specific (Fig. 9A, C, and E) or gC-specific (Fig. 9B, D, and F) monoclonal antibodies, followed by fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin G. Fluorescence microscopy demonstrated cell surface immunofluorescence for KOS-, gCB-, and gCΔ7B-infected Vero cells when the gD antibody was used. The level of fluorescence was similar for the three viruses, suggesting that the amount of gD expressed on the surface of cells infected with the two UL28 mutants is comparable to that in a wild-type infection. The

FIG. 6. Transmission electron micrographs of thin-section preparations of virus-infected cells. Vero cells infected with KOS (A), gCB (C), or gCΔ7B (E), C1 cells infected with KOS (B), gCB (D), or gCΔ7B (F). The arrowheads point to virus particles with or without an envelope. Abbreviations: N, nucleus; C, cytoplasm; E, extracellular.

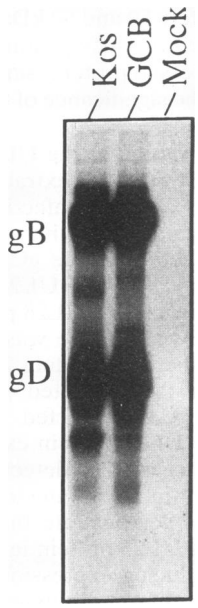

FIG. 8. Immunoprecipitation of gB and gD from Vero cells infected with KOS or gCB. Vero cells infected with the indicated virus were labeled with [^{35}S]methionine from 5 to 24 h postinfection, and gB and gD were immunoprecipitated with a mixture of gB- and gD-specific antibodies. Immunoprecipitated material was resolved by SDS-PAGE on 10% polyacrylamide gels and visualized by autoradiography.

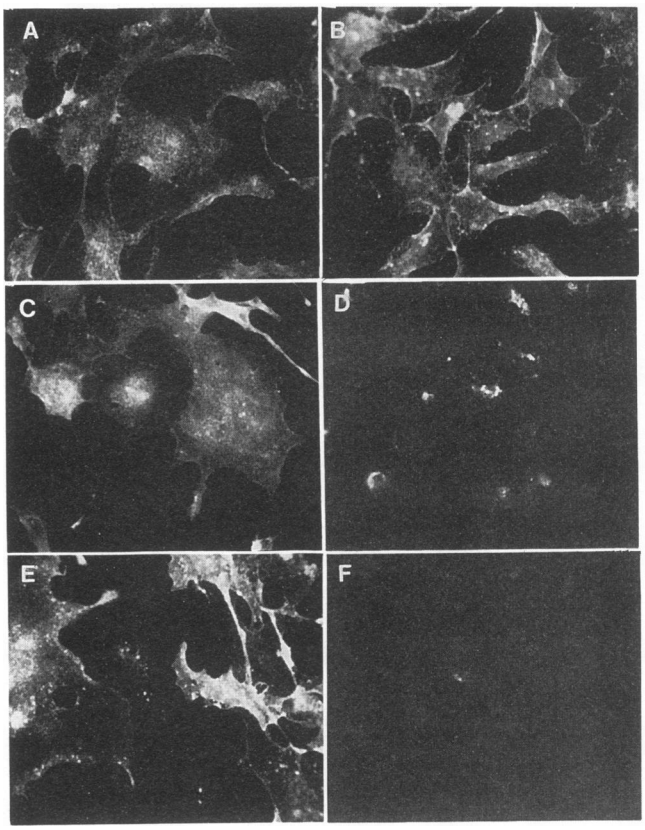

FIG. 9. Expression of viral glycoproteins on the surface of infected cells. Vero cells infected with KOS (A and B), gCB (C and D) or gCΔ7B (E and F) were fixed at 8 h postinfection with 3% paraformaldehyde and treated with gD-specific (panels A, C, and E) or gC-specific (panels B, D, and F) monoclonal antibodies followed by fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin G. Cell surface fluorescence was observed by fluorescence microscopy. The photographs in panels D and F were overexposed relative to the other panels to demonstrate the presence of cells.

parent virus used to construct the gCB and gCΔ7B mutants contains a deletion in the gC promoter, and so neither UL28 mutant expresses gC. When infected cells were treated with the gC antibody, cell surface immunofluorescence was observed only with wild type-infected cells. These data show that the UL28 gene product is not required for efficient expression of viral glycoproteins on the surface of infected cells.

DISCUSSION

Studies with HSV-1 *ts* mutants have demonstrated that cleavage of concatemeric HSV-1 DNA to unit-length molecules does not occur in the absence of DNA packaging (1–3, 39, 45, 46). In addition to the seven capsid proteins, at least five other viral gene (UL6, UL25, UL28, UL32, and UL33) products are required for the cleavage and packaging event (1–4, 46). Understanding the role of these genes in cleavage and packaging of viral DNA is important to our understanding of the mechanism of virus maturation. In this study we isolated Vero cell lines transformed with sequences that express the UL28 gene and used these cell lines to isolate two UL28 null mutants. Both of the UL28 null mutants isolated fail to form plaques on or replicate in Vero cells, confirming that this is an essential HSV-1 gene. Although we did not marker rescue the UL28 mutations, the fact that two different UL28 deletion mutants grow only on cell lines that express the UL28 gene strongly suggests that the phenotypes of gCB and gCΔ7B are due to the introduced mutations in the UL28 gene. The absence of expression of the UL28 gene product in Vero cells infected with the two mutants was demonstrated by immunoblot analysis with antiserum specific for this protein. The antiserum was also used in immunoblot experiments to show that in KOS-infected Vero cells, the UL28 gene product has an apparent molecular weight of 87,000, which is close to the molecular weight of 85,573 for the UL28 protein predicted by DNA sequence analysis (27), and that this protein is expressed at late times during infection. Electron microscopy and Southern blot analysis demonstrated that the UL28 null mutants are defective in cleavage and packaging of viral DNA.

A number of HSV *ts* mutants thought to have lesions in the UL28 gene have been isolated. The mutation in *ts*1203 has been mapped, by marker rescue, to the coding region of the UL28 gene. At the nonpermissive temperature, the phenotype of *ts*1203 is similar to that of the two UL28 null mutants described in this study, since this mutant produces only B capsids and fails to cleave high-molecular-weight viral DNA into unit-length molecules (2). In contrast to the UL28-null mutants, though, no cytoplasmic enveloped particles were detected in *ts*1203-infected cells at the nonpermissive temperature. The *ts*1203 mutant produces large numbers of intermediate capsids that are found only in the nucleus of infected cells, whereas Vero cells infected with either of the two UL28 null mutants contain nearly equal numbers of intermediate capsids in both the nucleus and the cytoplasm. Some of the particles found in the cytoplasm of

Vero cells infected with the UL28 null mutants contain a membrane envelope which, when examined by transmission electron microscopy, appears different from the envelope found in C1 cells infected with wild-type virus or with either of the two mutants. The membrane is very defined and appears to be missing the electron-opaque amorphous region beneath the envelope that is seen with wild-type virus particles (Fig. 6). This region may represent the tegument portion of the virus particle. To examine whether this portion of the virus particle is missing from the two null mutants, we plan to isolate enveloped virions from Vero cells infected with the two UL28 null mutants and examine their protein make-up by SDS-polyacrylamide gel electrophoresis (PAGE). There are several proteins associated with the tegument region, such as VP16, that are easily identified by SDS-PAGE, and these proteins can be used as markers to determine whether the tegument is present in UL28 mutant particles.

Studies with a second *ts* mutant, *ts*Z47, suggest that the UL28 gene product is needed for the efficient transport of viral glycoproteins to the cell membrane (31). These results are complicated by the fact that marker rescue studies have shown that *ts*Z47 contains two *ts* mutations (31), one that maps to the left end of the HSV-1 genome (map units 0.058 to 0.074) and one that maps to a region near the UL28 gene (map units 0.312 to 0.415). Furthermore, Addison et al. (2) have shown that in complementation tests *ts*1203 efficiently complements *ts*Z47, indicating that *ts*Z47 probably does not contain a lesion in UL28. To study the role of the UL28 gene product in glycoprotein transport, we examined the expression of gD on the surface of Vero cells infected with a UL28 null mutant. Immunofluorescence clearly demonstrated that a UL28 null mutation does not alter the expression of the gD gene on the surface of infected cells. We conclude from these studies that the UL28 gene product is not involved in the transport of viral glycoproteins to cell membranes. Studies with a pseudorabies virus mutant that contains a deletion in the UL28 gene homolog corroborate these results (30).

In summary, we have described a system for studying the role of the UL28 gene product in HSV-1 morphogenesis. UL28-transformed Vero cell lines which allow for the isolation and preparation of mutant virus stocks have been established. The availability of the complementing cell lines will allow for the isolation of additional UL28 mutants containing both deletion and nonsense mutations. Characterization of these mutants should ultimately lead to an understanding of the role of the UL28 protein in the cleavage and packaging of viral DNA. The fact that the UL28 gene is conserved among almost all herpesviruses indicates that this protein has a common function in all these viruses. Understanding how this protein functions during HSV-1 infections may therefore tell us how cleavage/packaging occurs in other herpesviruses.

ACKNOWLEDGMENTS

We thank Chris Fisher for help with the immunofluorescence studies and Kathy Kronke for preparation of the manuscript.

N.E.P. and P.R.S. were supported by Biomedical Research Support Grant 2 S07 RR05812-12.

REFERENCES

1. **Addison C., F. J. Rixon, J. W. Palfreyman, M. O'Hara, and V. G. Preston.** 1984. Characterization of a herpes simplex virus type 1 mutant which has a temperature-sensitive defect in penetration of cells and assembly of capsids. Virology **138**:246–259.
2. **Addison, C., F. J. Rixon, and V. G. Preston.** 1990. Herpes simplex virus type 1 UL28 gene product is important for the formation of mature capsids. J. Gen. Virol. **71**:2377–2384.
3. **Al-Kobaisi, M. F., F. J. Rixon, I. McDougall, and V. G. Preston.** 1991. The herpes simplex virus UL33 gene product is required for the assembly of full capsids. Virology **180**:380–388.
4. **Atkinson, M. A., S. Barr, and M. C. Timbury.** 1978. The fine structure of cells infected with temperature-sensitive mutants of herpes simplex virus type 1. J. Gen. Virol. **40**:103–119.
5. **Cai, W., S. Person, S. C. Warner, J. Zhou, and N. A. DeLuca.** 1987. Linker-insertion nonsense and restriction-site deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1. J. Virol. **61**:714–721.
6. **Cassai, E. N., M. Sarmiento, and P. G. Spear.** 1975. Comparison of the virion proteins specified by herpes simplex virus types 1 and 2. J. Virol. **16**:1327–1331.
7. **Cohen, G. H., M. Ponce de Leon, H. Diggelmann, W. C. Lawrence, S. Vernon, and R. J. Eisenberg.** 1980. Structural analysis of the capsid polypeptides of herpes simplex virus types 1 and 2. J. Virol. **34**:521–531.
8. **Davison, M. D., F. J. Rixon, and A. J. Davison.** 1992. Identification of genes encoding two capsid proteins (VP24 and VP26) of herpes simplex virus type 1. J. Gen. Virol. **73**:2709–2713.
9. **DeLuca, N. A., D. J. Bzik, V. C. Bond, S. Person, and W. Snipes.** 1982. Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion, and production of glycoprotein gB (VP7). Virology **122**:411–423.
10. **Desai, P., N. A. DeLuca, J. C. Glorioso, and S. Person.** 1993. Mutations in herpes simplex virus type 1 genes encoding VP5 and VP23 abrogate capsid formation and cleavage of replicated DNA. J. Virol. **67**:1357–1364.
11. **Dragan, D. J.** 1986. The structure and assembly of herpesviruses, p. 359–437. *In* J. R. Harris and R. W. Horne (ed.), Electron microscopy of proteins. Academic Press Ltd., London.
12. **Gadler, H.** 1983. Nucleic acid hybridization for measurement of effects of antiviral compounds on human cytomegalovirus DNA replication. Antimicrob. Agents Chemother. **24**:370–374.
13. **Gibson, W., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J. Virol. **10**:1044–1052.
14. **Gorman, C. R., R. Padmanabhan, and B. H. Howard.** 1983. High efficiency DNA-mediated transformation of primate cells. Science **221**:551–553.
15. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. 1973. Virology **52**:456–467.
16. **Heilman, C. J., Jr., M. Zwieg, J. R. Stephenson, and B. Hamper.** 1979. Isolation of a nucleocapsid polypeptide of herpes simplex virus types 1 and 2 possessing immunologically type-specific and cross-reactive determinants. J. Virol. **29**:34–42.
17. **Heine, J. W., R. W. Honess, E. N. Cassai, and B. Roizman.** 1974. Proteins specified by herpes simplex virus. XIII. The virion polypeptides of type 1 strains. J. Virol. **14**:649–651.
18. **Highlander, S. L., W. L. Sutherland, P. J. Gage, D. C. Johnson, M. Levine, and J. C. Glorioso.** 1987. Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. J. Virol. **61**:3356–3364.
19. **Holland, L. E., R. M. Sandri-Goldin, A. L. Goldin, J. C. Glorioso, and M. Levine.** 1984. Transcriptional and genetic analysis of the herpes simplex virus type 1 genome: coordinates 0.29–0.45. J. Virol. **49**:947–959.
20. **Holland, T. C., F. L. Homa, S. D. Marlin, M. Levine, and J. C. Glorioso.** 1984. Herpes simplex virus type 1 glycoprotein C-negative mutants exhibit multiple phenotypes, including secretion of truncated glycoproteins. J. Virol. **52**:566–574.
21. **Holland, T. C., S. D. Marlin, M. Levine, and J. Glorioso.** 1983. Antigenic variants of herpes simplex virus selected with glycoprotein-specific monoclonal antibodies. J. Virol. **45**:672–682.
22. **Homa, F. L., T. M. Otal, J. C. Glorioso, and M. Levine.** 1986. Transcriptional control signals of a herpes simplex virus type 1 late (γ_2) gene lie within bases −34 to +124 relative to the 5′ terminus of the mRNA. Mol. Cell. Biol. **6**:3652–3666.

23. **Johnson, G. D., and G. M. Nogueira Araujo.** 1981. A simple method for reducing the fading of immunofluorescence during microscopy. J. Immunol. Methods **43**:349–350.
24. **Liu, F., and B. Roizman.** 1991. The herpes simplex virus 1 gene encoding a protease also contains within its coding domain the gene encoding the more abundant substrate. J. Virol. **65**:5149–5156.
25. **Marlin, S. D., S. L. Highlander, T. C. Holland, M. Levine, and J. C. Glorioso.** 1986. Antigenic variation (*mar* mutations) in herpes simplex virus glycoprotein B can induce temperature-dependent alterations in gB processing and virus production. J. Virol. **59**:142–153.
26. **Marsden, H. S., I. K. Crombie, and J. H. Subak-Sharpe.** 1976. Control of protein synthesis in herpesvirus-infected cells: analysis of the polypeptides induced by wild type and sixteen temperature-sensitive mutants of HSV strain 17. J. Gen. Virol. **31**:347–372.
27. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531–1574.
28. **McNabb, D. S., and R. J. Courtney.** 1992. Identification and characterization of the herpes simplex virus type 1 virion protein encoded by the UL35 open reading frame. J. Virol. **66**:2653–2663.
29. **McNabb, D. S., and R. J. Courtney.** 1992. Posttranslational modification and subcellular localization of the p12 capsid protein of herpes simplex virus type 1. J. Virol. **66**:4839–4847.
30. **Mettenleiter, T. C., A. Saalmuller, and F. Weiland.** 1993. Pseudorabies virus protein homologous to herpes simplex virus type 1 ICP18.5 is necessary for capsid maturation. J. Virol. **67**:1236–1245.
31. **Pancake, B. A., D. P. Aschman, and P. A. Schaffer.** 1983. Genetic and phenotypic analysis of herpes simplex virus type 1 mutants conditionally resistant to immune cytolysis. J. Virol. **47**:568–585.
32. **Pederson, N. E., and L. W. Enquist.** 1991. Overexpression in bacteria and identification in infected cells of the pseudorabies virus protein homologous to herpes simplex virus type 1 ICP18.5. J. Virol. **65**:3746–3758.
33. **Pellett, P. E., F. J. Jenkins, M. Ackermann, M. Sarmiento, and B. Roizman.** 1986. Transcription initiation sites and nucleotide sequence of a herpes simplex virus type 1 gene conserved in the Epstein-Barr virus genome and reported to affect the transport of viral glycoproteins. J. Virol. **60**:1134–1140.
34. **Perdue, M. L., J. C. Cohen, M. C. Kemp, C. C. Randall, and D. J. O'Callaghan.** 1975. Characterization of three species of nucleocapsids of equine herpesvirus type 1 (EHV-1). Virology **64**:187–204.
35. **Perdue, M. L., J. C. Cohen, C. C. Randall, and D. J. O'Callaghan.** 1976. Biochemical studies of the maturation of herpes virus nucleocapsid species. Virology **74**:194–208.
36. **Perdue, M. L., M. C. Kemp, C. C. Randall, and D. J. O'Callaghan.** 1974. Studies of the molecular anatomy of the L-M cell strain of equine herpes virus type 1: proteins of the nucleocapsid and intact virion. Virology **59**:201–216.
37. **Pertuiset, B., M. Boccara, J. Cebrian, N. Berthelot, S. Chousterman, F. Puvion-Dutilleul, J. Sisman, and P. Sheldrick.** 1989. Physical mapping and nucleotide sequence of a herpes simplex virus type 1 gene required for capsid assembly. J. Virol. **63**:2169–2179.
38. **Powell, K. L., and D. H. Watson.** 1975. Some structural antigens of herpes simplex virus type 1. J. Gen. Virol. **29**:167–177.
39. **Preston, V. G., J. A. Coates, and F. J. Rixon.** 1983. Identification and characterization of a herpes simplex virus gene product required for encapsidation of virus DNA. J. Virol. **45**:1056–1064.
40. **Preston, V. G., F. J. Rixon, I. M. McDougall, M. McGregor, and M. F. Al-Kobaisi.** 1992. Processing of the herpes simplex virus assembly protein ICP35 near its carboxy terminal end requires the product of the whole of the UL26 reading frame. Virology **186**:87–93.
41. **Rixon, F. J., M. D. Davison, and A. J. Davison.** 1990. Identification of the genes encoding two capsid proteins of herpes simplex virus type 1 by direct amino acid sequencing. J. Gen. Virol. **71**:1211–1214.
42. **Roizman, B.** 1990. Herpesviridae: a brief introduction, p. 1787–1793. *In* B. N. Fields (ed.), Virology. Raven Press, New York.
43. **Schaffer, P. A., G. M. Aron, N. Biswal, and M. Benyesh-Melnick.** 1973. Temperature-sensitive mutants of herpes simplex virus type 1: isolation, complementation and partial characterization. Virology **52**:57–71.
44. **Schaffer, P. A., J. P. Brunschwig, R. M. McCombs, and M. Benyesh-Melnick.** 1974. Electron microscopic studies of temperature-sensitive mutants of herpes simplex virus type 1. Virology **62**:444–457.
45. **Sherman, G., and S. L. Bachenheimer.** 1987. DNA processing in temperature-sensitive morphogenic mutants of HSV-1. Virology **158**:427–430.
46. **Sherman, G., and S. L. Bachenheimer.** 1988. Characterization of intranuclear capsids made by ts morphogenic mutants of HSV-1. Virology **163**:471–480.
47. **Spear, P. G.** 1980. Composition and organization of herpesvirus virions and properties of some of the structural proteins, p. 54–78. *In* F. Rapp (ed.), Oncogenic herpesviruses, vol. 1. CRC Press, Inc., Boca Raton, Fla.
48. **Spear, P. G., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. **9**:143–159.
49. **Stow, N. D., E. C. McMonagle, and A. J. Davison.** 1983. Fragments from both termini of the herpes simplex virus type 1 genome contain signals required for the encapsidation of viral DNA. Nucleic Acids Res. **11**:8205–8220.
50. **Weller, S. K., E. P. Carmichael, D. P. Aschman, D. J. Goldstein, and P. A. Schaffer.** 1987. Genetic and phenotypic characterization of mutants in four essential genes that map to the left half of HSV-1 UL DNA. Virology **161**:198–210.
51. **Wildy, P.** 1986. Herpesvirus. Intervirology **25**:117–140.
52. **Zhu, L., and S. K. Weller.** 1992. The UL5 gene of herpes simplex virus type 1: isolation of a *lacZ* insertion mutant and association of the UL5 gene product with other members of the helicase-primase complex. J. Virol. **66**:458–468.